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Genetic analysis of morphological variants of *Aspergillus parasiticus* deficient in secondary metabolite production

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Aflatoxins (AFs) are secondary metabolites produced mainly by *Aspergillus parasiticus* and *A. flavus*. To study AF regulation, previously isolated non-toxigenic *A. parasiticus sec*− (for secondary metabolism minus) variants were genetically analysed. In parasexual crossing, the *sec*− strains failed to form heterokaryons and diploids with other *sec*− strains. Heterokaryon test results suggested that involvement of cytoplasmic elements in the formation of *sec*− phenotype was unlikely. At the molecular level, the coding sequence of the *sec*− *afl*R (the only known positive regulator of AF pathway) was identical to that of their toxigenic *sec*+ (for secondary metabolism plus) parents. However, the *sec*− *afl*R expression was 5- to 10-fold lower compared to that in the *sec*+ forms. RT–PCR analysis demonstrated that the AF pathway genes were expressed in the *sec*− forms but in trace amounts and in their unprocessed forms. Combined, these results suggest that *afl*R is necessary but not sufficient for AF production and that elements involved in fungal development directly or indirectly influence its proper function.

INTRODUCTION

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced mainly by three anamorphic species of the genus *Aspergillus*: *A. flavus*, *A. parasiticus*, and *A. nomius*. Initially studied because of their negative impact on human and animal health (Eaton & Gallagher 1994), aflatoxins have also emerged as a model system for studying the genetics of mycotoxin biosynthesis and secondary metabolism (Skory *et al.* 1992, Chang *et al.* 1993, Cary, Linz & Bhatnagar 2000, Bhatnagar, Ehrlich & Cleveland 2003).

Classical aflatoxin (AF) biosynthesis has been studied using a combination of blocked mutants, isotopic labelling and biofeeding experiments (Bhatnagar, Ehrlich & Cleveland 1992, Minto & Townsend 1997, Payne & Brown 1998). Important contributions have also been made on a related sterigmatocystin (ST) pathway in *Aspergillus nidulans* where ST, the ultimate product in *A. nidulans*, is a late precursor of AF production in *A. flavus* and *A. parasiticus* (Brown *et al.* 1996, Butchko, Adams & Keller 1999). Results of many such refined studies have demonstrated that genes for both AF, in the AF biosynthetic pathway, and for ST in the ST biosynthetic pathway are clustered on one chromosome (Trail *et al.* 1995, Yu *et al.* 1995, Bhatnagar *et al.* 2003), and comparison of the AF cluster to the ST cluster has revealed a high degree of similarity with respect to the gene function and structure, although the order of the genes is not as highly conserved (Yu *et al.* 1995, Bhatnagar *et al.* 2003).

The regulatory aspects of AF biosynthesis are less well understood. To date, only one gene has been shown to be essential for the expression of the pathway structural genes. That gene, designated *afl*R (originally called *afl-2* in *A. flavus*; Payne *et al.* 1993; and *apa-2* in *A. parasiticus*; Chang *et al.* 1993), encodes a zinc bi-nuclear cluster-type, sequence-specific DNA-binding protein. The function of *afl*R has been conserved in the ST pathway as well (Yu *et al.* 1996).

We have studied another unusual aflatoxin-negative phenotype which we have named *sec*− (for 'secondary metabolism minus') (Kale *et al.* 1994, 1996). A collection of six variant *sec*− strains were isolated after serial transfers of non-sporulating mycelial macerates of the respective *sec*+ (for secondary metabolism plus) parental forms. These highly stable *sec*− strains exhibited a pleiotropic effect in which loss of AF production was correlated with phenotypic alterations in morphology, specifically conidiation (Kale *et al.* 1994).

Table 1. Aflatoxin and/or anthraquinone production by strains used in the parasexual analysis.

Strain[a]	Norsolorinic acid (ng) ($\times 10^0$)	Versicolorin A (ng) ($\times 10^0$)	Aflatoxin (ng) ($\times 10^3$)			
			B1	B2	G1	G2
1. *wh-1 pdx-2*	ND[b]	623	700	73	63	3.7
2. *br-1 pdx-1*	ND	TR[c]	110	70	0.4	0.6
3. *wh-1 nor-1 lys-6 ade-1*	368	ND	105	43	9.8	0.8
4. *br-1 nor-1 lys-6 ade-1*	426	ND	25	10	TR	TR
5. *wh-1 ver-1 lys-6 pdx-1*	ND	920	ND	ND	ND	ND
6. *br-1 met-1*	TR	64	TR	TR	ND	ND
7. *wh-1 nor-1 ade-1*	179	ND	TR	TR	ND	ND
8. *br-1 nor-1 lys-5*	62	ND	ND	ND	ND	ND
9. *br-1 nor-1 met-1*	112.6	ND	TR	TR	ND	ND
10. *wh-1 ver-1 lys-7*	ND	176	ND	ND	ND	ND

[a] Strains 1–5 were the parental strains used in previous studies to obtain the *sec*− forms (Kale *et al*. 1994, 1996). Strains 6–10 were used as the crossing strains in this study. None of the *sec*− variants produced any detectable levels of aflatoxins and/or pathway intermediates, hence are not included in this table.

[b] ND, none detected.

[c] TR, trace amounts (< 10 ng).

When fed with labelled precursors, the *sec*− forms failed to bioconvert the precursors to AF, although Southern blot and polymerase chain reaction (PCR) analyses revealed that the AF pathway genes were intact (Kale *et al*. 1996). In addition, Northern blot experiments of the *sec*− forms suggested a lack of detectable expression of both the AF pathway structural genes and the *afl*R gene (Kale *et al*. 1996).

In this study, we have used both classical and molecular genetic approaches to further analyse this highly intriguing class of non-toxigenic *sec*− variants. Specifically, we have focused our attention on (1) the behaviour of the *sec*− spores in parasexual crosses with respect to formation of heterokaryons and diploids, and (2) the *afl*R gene with respect to its DNA sequence and transcript levels in the *sec*− strains, using both traditional Northerns and the more sensitive RT–PCR technique to ascertain the degree of involvement of this regulatory gene in the formation of the *sec*− phenotype.

MATERIALS AND METHODS

Fungal strains and culture conditions

The fungal strains used in this study are listed in Table 1. One wild-type and five auxotrophic and spore colour *sec*+ mutants of *Aspergillus parasiticus* and the corresponding *sec*− forms isolated from the *sec*+ parents had the following genotypes: *SU-1*, *wh-1 pdx-2*, *br-1 pdx-1*, *wh-1 nor-1 lys-6 ade-1*, *br-1 nor-1 lys-6 ade-1* and *wh-1 ver-1 lys-6 pdx-1*, where *wh* = white spores, *pdx* = pyridoxine, *br* = brown spores, *nor* = norsolorinic acid (early precursor of AF), *ade* = adenine, *ver* (versicolorin A, precursor of AF) and *lys* = lysine. The geneologies of these *sec*+ forms and the isolation of the respective *sec*− derivatives have been described earlier in greater detail (Kale *et al*. 1994). In addition, the following *A. parasiticus* strains were used as the 'crossing parents' in the parasexual experiments: *br-1 met-1*, *wh-1 nor-1 ade-1*, *br-1 nor-1 lys-5*, *br-1 nor-1 met-1* and *wh-1 ver-1 lys-7*, where *met* = methionine. These six strains were obtained from the culture collection of J.W.B. (Tulane University, LA) and John F. Peberdy (University of Nottingham, Nottingham). All cultures were incubated at 30 °C in the dark; the cultures in the liquid media were maintained on a gyratory shaker (New Brunswick Scientific model G76) operating at 150 rpm.

Media and chemicals

Complete medium (CM; potato dextrose agar plus 0.5% yeast extract, Difco) was used for maintaining the stock cultures, viable counts, and analysis of segregants from diploids. Aflatoxin-producing agar (APA; Hara, Fennell & Hesseltine 1974) was used as a preliminary screen for aflatoxin-producing colonies by detection of blue–green fluorescence after 5 d incubation. Minimal medium (MM, Czapek's solution agar, Difco) was used for the isolation of auxotrophic mutants and for maintaining stock cultures of heterokaryons and diploids. To verify the diploidy, benomyl (0.5 ppm) was added to CM to induce haploidization. Nutritional supplements were added to MM in the following concentration: lysine and methionine, 250 mg l^{-1}; adenine, 20 mg l^{-1}; pyridoxine, 0.1 mg l^{-1}. For aflatoxin and anthraquinone assays, the liquid defined medium (AM) devised by Adye & Mateles (1964) was used with the substitution of 50 g sucrose/l for glucose. For isolation of RNA, cultures were grown on yeast extract sucrose (YES) medium (Filtenborg, Frisvad & Thrane, 1990).

Parasexual analysis

Formation of heterokaryons and diploids

Heterokaryons were made by mixing two loopfuls of spores from pairs of parental strains with contrasting

spore colour and nutritional markers on 1.5 ml of deionized water overlaying 6 ml of CM in a 15 ml capacity tube (Scott, Marascalco & Bennett 1979). For the *sec*− strains, the stock cultures were flooded with 0.85% saline and the spores concentrated by table-top centrifugation. In addition, since the *sec*− forms had reduced sporulation, 4–6 loopfuls of the concentrated *sec*− spores were used in each cross. For the *sec*+ forms, it was not necessary to concentrate the spores. After 24–48 h, mycelial mats were removed from the water surface, blotted dry on sterile filter paper, and shredded into five fragments, which were placed on MM. After 2 wk of incubation, presumptive heterokaryons were observed as irregularly shaped colonies consisting of a mixture of the component spore colour mutants. The heterokaryons were flooded with 0.85% saline and 0.5% Tween 80, and aliquots of the resulting dense spore suspension were plated on fresh MM plates to screen for presumptive diploids. Diploids were recognized as green colonies in the heterokaryotic background, purified by subculturing on fresh MM plates and single spore colonies were obtained by serial dilution technique. Stock cultures of diploids were prepared from single spore isolates on MM slants.

Heterokaryon (*cytoplasmic inheritance*) *test*

The heterokaryon plates with specific markers (spore colour and mycelial colour) were flooded with 0.85% saline and 0.5% Tween 80. The resulting thick spore suspension was then subjected to a series of dilutions. Aliquots of higher dilutions (10^{-5} to 10^{-6}) were plated on CM and the resulting colonies scored for the segregation of the specific spore colour and anthraquinone markers between the two heterokaryon components. For aflatoxin-producing species, the colonies from CM were subcultured on APA medium (Hara *et al*. 1974) and checked for fluorescence production after 5 d under long wave UV. Fluorescent colonies were considered positive for aflatoxin production.

Aflatoxin and anthraquinone assays

Strains were cultured in quadruplicate (haploids) or duplicate (diploids) in 100 ml AM flasks for 7 d at 150 rpm. The extraction procedure for secondary metabolites has been described in detail in Kale *et al*. (1996). The extracted dried samples containing the secondary metabolites were suspended in 1 ml of methylene dichloride and thin-layer chromatography (TLC) was carried out by spotting 15 µl of each sample and known standards on prescored 250 µm-thick silica gel G plates (20 × 20 cm, Analtech). The plates were developed with ether:methanol:water (96:3:1, by volume) for AF and ST (R_f = 0.44 for AFB1 and 0.97 for ST) and toluene:ethyl acetate:acetic acid (50:30:4, by volume) for anthraquinones (R_f = 0.73 for norsolorinic acid, 0.59 for averantin, and 0.64 for versicolorin A). The plates were dried and viewed under long-wave UV light for fluorescent metabolites.

Based on the initial TLC, visual estimates of the appropriate amounts of extracts to be spotted for densitometric analysis were obtained, as recommended by the American Oil Chemists' Society (Walker 1983). Accordingly, prescored 250 µm-thick silica gel G plates were spotted, developed in appropriate solvent systems, and scanned for fluorescence with a Shimadzu model CSW 9-10 recording densitometer at an excitation wavelength of 360 nm for AF, 310 nm for norsolorinic acid, and 290 nm for averantin and versicolorin A. The quantities of these metabolites were calculated by comparison with areas under the peaks for standards run on the same plate. Quantities of standards and samples were also verified spectrophotometrically (Shimadzu UV-visible UV-160 spectrophotometer) with known extinction coefficients (Cole & Cox 1981).

Nucleic acid isolation and analysis

DNA was purified from representative *Aspergillus parasiticus* strains by the method of Horng *et al*. (1990). Total fungal RNA was prepared by the hot-phenol method (Maramatsu 1973) and purified using the Qiagen RNeasy Plant Mini Kit (Qiagen) for Northern blotting and RT–PCR. Total mycelial RNA was separated on a 2.2 M formaldehyde–1% agarose gel and vacuum transferred to Nytran Plus membranes. The filters were prehybridized and hybridized in ULTRAhyb buffer (Ambion, Austin, TX) and probed with a ^{32}P-dCTP labelled DNA representing the *afl*R or *ver-1* or *omt*A gene coding regions. As an RNA loading control the constitutively expressed *nmt-1* gene (Cary & Bhatnagar 1995) was used as a probe. Filters were hybridized overnight at 42 ° then washed twice at 42 ° for 10 min each in 2 × SSC/1% SDS, followed by two additional washes for 15 min each at 42 ° in 0.1 × SSC/1% SDS. The filters were placed on Kodak BioMax film (Eastman Kodak, Rochester, NY) and allowed to expose for the desired time with intensification at −80 °. The *afl*R gene coding region of both *sec*+ and *sec*− *A. parasiticus* isolates was amplified from about 500 ng genomic DNA using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and the following oligonucleotide primers:

5′ *afl*R-ATGGTTGACCATATCTCCCCCCGGGCAT
3′ *afl*R-TCATTCTCGATGCAGGTAATCGATAATG

Thermocycler (PTC 100; MJ Research Inc., Watertown, MA) parameters were as follows: 1 cycle of 95 °, 10 min; 60 °, 1 min; 72 °, 1 min; 34 cycles of 95 °, 30 s; 65 °, 1 min; 72 °, 1 min; followed by a final extension at 72 ° for 7 min.

RT–PCR of total RNA was performed using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). RNase-Free DNase (Qiagen) was used to ensure

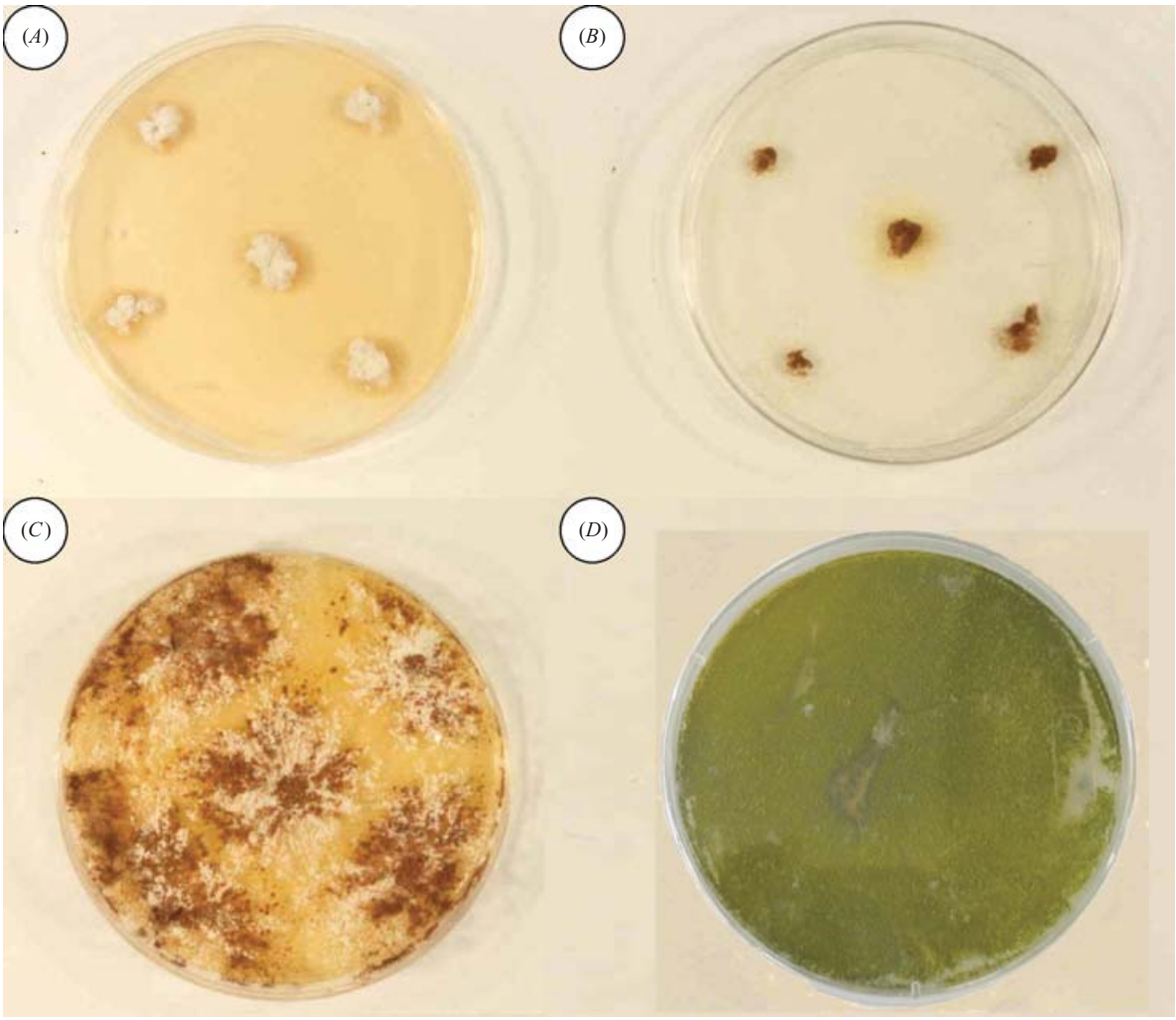

Fig. 1. Formation of a heterokaryon between two strains of *Aspergillus parasiticus* differing in spore colour and auxotrophic markers: (*A*) Strain *br-1 nor-1 lys-6 ade-1 sec+* on minimal medium (MM); (*B*) strain *wh-1 pdx-2 sec+* on MM, neither strain by itself can grow on MM because of auxotrophy; (*C*) a heterokaryon formed between these strains is able to grow on the MM and is seen as a mixture of both spore colours of both strains (brown and white respectively); and (*D*) a purified diploid from the above-mentioned cross has green spores. However, the shade of green is lighter than the wild-type *SU-1* strain. For additional details, see text.

complete removal of DNA from RNA samples prior to RT–PCR. Primer pairs for PCR reactions are given in Fig. 2. Five µl of cDNA template was amplified using the following thermocycler parameters: 1 cycle of 95 °, 10 min; 65 °, 1 min; 72 °, 30 s; 34 cycles of 95 °, 30 s; 68 °, 1 min; 72 °, 30 s; followed by a final extension at 72 ° for 7 min. The PCR products were sequenced using an ABI Prizm 377 Automated DNA Sequencer (Applied Biosystems). Sequence information was analysed using DNAMAN (Lynon Biosoft, Quebec) analysis software.

RESULTS

Parasexual cycle analysis

Formation of heterokaryons and diploids

The process of formation of a heterokaryon and the resulting diploid is shown in Fig. 1. A total of twenty-five parasexual crosses (9 *sec+ x sec+*, 11 *sec+ x sec−*, and 5 *sec− x sec−*) were attempted, as shown in Table 2. For the sake of convenience, since the crossing parents made either AF and/or pathway intermediates, they were also referred to as *sec+* strains. In all cases of the *sec+ x sec+* and *sec+ x sec−* crosses, presumptive heterokaryons were obtained, but their vigour varied (Table 2). In general, crosses involving both parents with the *sec+* phenotype yielded vigourously growing heterokaryons, while those with one of the parents as a *sec−* strain, especially *wh-1 ver-1 lys-6 pdx-1 sec−* and *wh-1 nor-1 lys-6 ade-1 sec−*, gave rise to poorly growing heterokaryons, as shown in Table 2. No crosses involving both partners as *sec−* strains (e.g. *wh-1 pdx-2 sec− x br-1 nor-1*, *lys-6*, *ade-1 sec−*) resulted in formation of a heterokaryon even after 10 attempts (Table 2).

Twelve diploids were isolated from the twenty-four crosses. The presumptive diploids were observed as

Table 2. Attempted parasexual crosses using contrasting spore colour and auxotrophic markers.

Cross[a]	Component strains	Heterokaryon formation[d]	Diploid isolation	Number of attempts
I. *sec+ x sec+*[a]				
1	*wh-1 pdx-2 sec+ x br-1 nor-1 lys-6 ade-1 sec+*	++++	Yes	1
2	*wh-1 pdx-2 sec+ x br-1 nor-1 lys-5 sec+*	++++	Yes	1
3	*wh-1 pdx-2 sec+ x br-1 nor-1 met-1 sec+*	++++	Yes	1
4	*wh-1 ver-1 lys-6 pdx-1 sec+ x br-1 met-1 sec+*	++	Yes	2
5	*wh-1 ver-1 lys-6 pdx-1 sec+ x br-1 nor-1 met-1 sec+*	+++	Yes	2
6	*wh-1 nor-1 lys-6 ade-1 sec+ x br-1 pdx-1 sec+*	++	Yes	4
7	*wh-1 nor-1 lys-6 ade-1 sec+ x br-1 met-1 sec+*	+++	Yes	2
8	*br-1 pdx-1 sec+ x wh-1 ver-1 lys-7 sec+*	++++	Yes	1
9	*br-1 pdx-1 sec+ x wh-1 nor-1 ade sec+1*	+++	No	5
II. *sec+ x sec−*[b]				
10	*wh-1 pdx-2 sec− x br-1 nor-1 lys-6 ade-1 sec+*	+++	Yes	2
11	*wh-1 pdx-2 sec− x br-1 nor-1 lys-5 sec+*	+++	Yes	1
12	*wh-1 pdx-2 sec− x br-1 nor-1 met-1 sec+*	+++	Yes	1
13	*wh-1 ver-1 lys-6 pdx-1 sec− x br-1 met-1 sec+*	+	No	5
14	*wh-1 ver-1 lys-1 pdx-1 sec− x br-1 nor-1 met-1 sec+*	+	No	5
15	*wh-1 nor-1 lys-6 ade-1 sec− x br-1 pdx-1 sec+*	+	No	4
16	*wh-1 nor-1 lys-6 ade-1 sec− x br-1 met-1 sec+*	+	No	5
17	*br-1 pdx-1 sec− x wh-1 ver-1 lys-7 sec+*	++	No	4
18	*br-1 pdx-1 sec− x wh-1 nor-1 lys-6 ade-1 sec+*	+++	Yes	2
19	*br-1 pdx-1 sec− x wh-1 nor-1 ade-1 sec+*	+	No	4
20	*br-1 nor-1 lys-6 ade-1 sec− x wh-1 pdx-1 sec+*	++	No	5
III. *sec− x sec−*[c]				
21	*wh-1 pdx-1 sec− x br-1 nor-1 lys-6 ade-1 sec−*	No	No	10
22	*br-1 pdx-1 sec− x wh-1 nor-1 lys-6 ade-1 sec−*	No	No	10
23	*wh-1 pdx-1 sec− x br-1 met-1 sec−*	No	No	10
24	*wh-1 nor-1 lys-6 ade-1 sec− x br-1 met-1 sec−*	No	No	10

[a] As mentioned in the text, the term '*sec+*' represents strains, which make aflatoxins and/or pathway intermediates (Table 1). These include those used in previously (Kale *et al.* 1994) plus the crossing parents from this study.

[b] The *sec−* variants do not make any detectable levels of aflatoxins or pathway intermediates.

[c] The *sec−* variant of *br-1 met-1* was isolated using the previously described procedure (Kale *et al.* 1994) and was specifically used in the *sec− x sec−* crosses to increase the total number of crosses in this category.

[d] Growth of the heterokaryons on MM plates expressed on a scale of + to ++++ with abundant growth of evenly distributed parental spores represented as ++++.

small, discrete colonies of green coloured spores among a lawn of parental brown, white and/or yellow spore colours. They were subsequently purified on MM and confirmed as diploids by their ability to grow and give rise to haploid segregants showing the recessive parental phenotypic markers in the presence of benomyl. Eight of the twelve diploids were derived from *sec+ x sec+* crosses, while four were isolated from *sec+ x sec−* crosses. In general, crosses yielding healthy heterokaryons formed diploids with relative ease (after one or two attempts). In contrast, most crosses showing intermediate to poor quality heterokaryons (Table 2) did not yield any diploids even after five or six attempts. In the *sec+ x sec−* crosses, with the exception of *wh-1 pdx-2 sec−*, all other *sec−* strains failed to form good heterokaryons and consequently no diploids were isolated. This was true even upon centrifuging and concentrating the *sec−* spores and using 4–6 loopfuls (compared to 2 loopfuls of unconcentrated *sec+* spores) and after several attempts (Table 2). As stated above, none of the *sec− x sec−* crosses resulted in formation of heterokaryons and consequently, diploids.

Aflatoxin and/or anthraquinone production by the *Aspergillus parasiticus* haploid and diploid strains used in the parasexual crosses are shown in Tables 1 and 3. All twelve diploids produced secondary metabolites in the defined liquid medium. With one exception (diploid 1), all the diploids made more aflatoxin than either of their parental components. Further, most *sec+ x sec−* diploids (e.g. 10, 11, 12, 18) produced aflatoxins in similarly high levels as the *sec+ x sec+* diploids (e.g. 1, 2, 3, 6), in a range of 330–940 μg of AFB1 in 100 ml of defined medium (Table 3).

Heterokaryon test

All heterokaryons resulting from *sec+ x sec−* crosses were subjected to the heterokaryon test. In each case, the fused heterokaryotic mycelium did resolve into its component homokaryons. However, in each heterokaryon, the pleiotropic *sec−* phenotype (altered morphology, reduced sporulation and loss of secondary metabolite production) remained associated with the spore colour (nuclear marker) of the *sec−* partner. In other words, this test was negative and no evidence of

Table 3. Aflatoxin and anthraquinone production by *Aspergillus parasiticus* diploids (average of four 100 ml AM medium flasks for each diploid).

Diploid number[a]	Nor (ng) ($\times 10^0$)	Ver A (ng) ($\times 10^0$)	AF (ng) ($\times 10^3$)			
			B1	B2	G1	G2
1	TR[b]	152	539	1526	ND[c]	30
2	TR	98	941	2674	ND	80
3	TR	277	429	501	TR	TR
4	TR	101	554	1434	ND	32
5	TR	221	300	224	98	TR
6	TR	239	365	437	TR	TR
7	TR	TR	400	1061	ND	19
8	TR	TR	4438	662	46	10
10	TR	ND	333	1042	ND	9.2
11	TR	ND	593	1759	ND	34
12	TR	ND	431	1033	ND	14
18	TR	TR	632	1773	ND	51

[a] The diploid number corresponds to the cross number in Table 2.
[b] TR, trace amounts (< 10 ng).
[c] ND, none detected.

recombination was detected in any of the *sec*+ *x sec*− heterokaryons (data not shown).

Nucleic acid analysis

Afl*R sequencing*

The coding sequence for the *afl*R gene from *sec*+ and *sec*− strains was identical and no difference was detected by the DNAMAN analysis. Thus, the *sec*− sequence was identical to that of the *apa-2* (original name of *afl*R) gene of *A. parasiticus* which was deposited in GenBank (accession no. L22177) by Chang *et al*. (1993).

Northern blot analysis

Total RNA from four representative strains (*SU-1 sec*+, *SU-1 sec*−, *br pdx sec*+, and *br pdx sec*−) was analysed by Northern blot hybridization. Comparison of *afl*R transcript levels in the *sec*+ and the *sec*− strains revealed that while *afl*R was being expressed in all strains tested, the levels of the *afl*R transcript were approximately 5- to 10-fold lower in the *sec*− variants (Fig. 2*A*). Hybridization of the total RNA with a probe specific for the aflatoxin pathway structural genes *omt*A and *ver-1* showed that both transcripts were present in the *sec*+ strains, while neither transcript could be detected in the *sec*− RNA.

RT–PCR analysis

RT–PCR analysis of *afl*R, two aflatoxin biosynthetic genes (*ver-1* and *omt*A), and one constitutively expressed housekeeping gene (*nmt-1*) from the four fungal strains was also performed (Fig. 2*B*). PCR primers to the *ver-1*, *omt*A, and *nmt-1* genes were designed such that at least one intron of the gene coding region would be amplified in addition to the adjacent exons (*afl*R gene does not have introns). Thus, any contamination of the template RNA by DNA would be identifiable by the presence of a larger PCR product due to the presence of the intron sequence. The expected size amplification products were obtained upon PCR of the cDNAs from all strains with respect to *afl*R. The PCR of the *sec*+ cDNAs with *ver-1*, *omt*A and *nmt-1* primers gave the expected size products, with the cDNA products being slightly smaller than the genomic products due to the processing of the introns. However, amplification of the *sec*− cDNAs resulted in PCR products that were the same size as the genomic DNA products except for the *nmt-1* gene control product that was the expected size for the cDNA. The presence of a PCR product that represents *nmt-1* cDNA only in all strains tested indicated that there was no genomic DNA contamination present in the template cDNAs.

DISCUSSION

The parasexual cycle (Roper 1966) is the only known natural mechanism of genetic recombination in the imperfect fungi. It consists of anastomosis (fusion) of parental hyphae to form a heterokaryon in which the two parental nuclei co-exist as separate entities, followed by fusion of the parental nuclei to form a heterozygous diploid. Haploidization and/or mitotic recombination in the diploid may form recombinant progeny (Bennett 1979). In a laboratory setting, agents such as benlate (Hastie 1970) are often used to expedite the process of haploidization and consequently, the isolation of segregants. Over the past few decades, parasexual analysis has been extensively used in those imperfect fungi which produce metabolites of commercial and/or economic importance. Such applications have included characterizing stable heterozygous diploids of the koji- moulds *Aspergillus oryzae* and *A. sojae* (Ishitani, Ikeda & Sakaguchi 1958), isolating high penicillin-yielding recombinant strains of *Penicillium chrysogenum* (Ball 1982), analysing stable intergeneric diploids of *Monascus purpurens* (syn. *M. anka*) and *A. oryzae* to identify populations of diploids producing ethanol earlier than either parents (Kiyohara *et al*. 1990) and more recently, analysing protoplast-fusion products (Couteaudier, Viaud & Riba 1996, Ogawa *et al*. 2000). With respect to the aflatoxigenic aspergilli, the parasexual cycle was employed to establish eight linkage groups in *A. flavus* (Papa 1976, 1979), and six in *A. parasiticus* (Bradshaw, Bennett & Peberdy 1983).

Earlier we had shown that *A. parasiticus sec*− variants were non-toxigenic, stable, and morphologically altered (Kale *et al*. 1994). Further, we had shown that the AF pathway genes were present in these *sec*− strains, even though they failed to bioconvert AF precursors in the biofeeding studies (Kale *et al*. 1996).

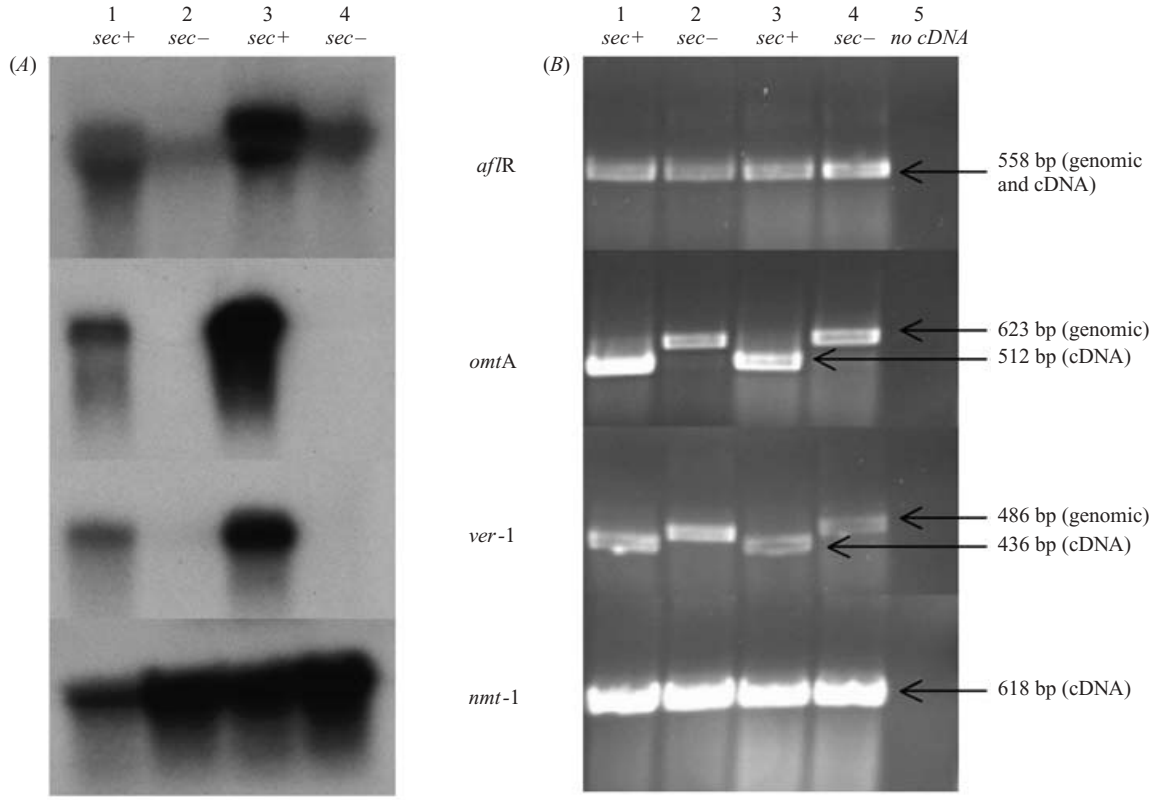

Fig. 2. (*A*) Composite of Northern blot analysis of representative *Aspergillus parasiticus sec*+ and *sec*− strains. Total RNA from 48 h-old cultures of YES-grown fungal mycelia was purified, electrophoresed, transferred to nylon membranes and hybridized with radiolabelled fragments of the *afl*R, *omt*A, or *ver-1* coding regions. As an RNA loading control the *nmt-1* (Cary & Bhatnagar 1995) housekeeping gene was used as a probe. The RNAs analysed are as follows: lanes (1) *SU-1 sec*+; (2) *SU-1 sec*−; (3) *br-1 pdx-1 sec*+; (4) *br-1 pdx-1 sec*−. (*B*) Composite of ethidium bromide stained RT–PCR products from representative *A. parasiticus sec*+ and *sec*− strains. Lanes: (1) *br-1 pdx-1 sec*+; (2) *br-1 pdx-1 sec*−; (3) *SU-1 sec*+; (4) *SU-1 sec*−; (5) no cDNA control.

Primer pairs used and expected size of PCR products were:

5′ *afl*R (TCATTCTCATCCGCAACCGCATCCACA) and 3′ *afl*R (AAGTAGCCATCCTGCGCGCACGAACA), 558 bp genomic and cDNA (since *afl*R lacks introns); 5′ *ver-1* (CACCGTTTAGATGGCAAAGTGGCCTTG) and 3′ *ver-1* (TTGACGCAAGCGGTGTTAGAGCTGGTC), 486 bp genomic, 436 cDNA; 5′ *omt*A (CAAAGATGTTGCGAGTGACGGGCATTCA) and 3′ *omt*A (CGGGATAGTCATGTAGAATAGATCGGA), 623 bp genomic, 512 bp cDNA; 5′ *nmt-1* (GCTTCAAGGCCATGATCCATACTCTG) and 3′ *nmt-1* (ACCCAGACGCTTTCCGTAGTTGGTA), 672 bp genomic, 618 cDNA.

In the current study, we conducted parasexual cycle analysis for genetically characterizing the *sec*− strains and for investigating the probable cause(s) of formation of the *sec*− phenotype, including the possible involvement of cytoplasmic elements.

The results of our twenty-five crossing experiments showed that all *sec*+ *x sec*+ and *sec*+ *x sec*− crosses yielded heterokaryons of varying vigour, while none of the *sec*− *x sec*− crosses resulted in heterokaryon formation (Table 2). The parasexual results (Tables 2 and 3) also demonstrated clear differences between the *sec*− strains and a related non-toxigenic *afl-1 A. flavus* mutant (Papa 1980). For example, the *afl-1* diploids have been shown to be dominant negative (Woloshuk *et al.* 1995), while the *sec*− diploids are recessive to their *sec*+ partners (Table 3). Also, while Papa's *afl-1* strain is a product of parasexual crossing (Papa 1980), the *sec*− strains are products of sequential transfers of macerated mycelia (Kale *et al.* 1994). Further, it has been shown that the *afl-1* mutant is a result of deletion of a large segment of a chromosome which includes several AF pathway genes (Woloshuk *et al.* 1995); whereas the entire AF biosynthetic pathway appears to be intact in the *sec*− strains (Kale *et al.* 1996).

The heterokaryon test establishes the difference between nuclear versus cytoplasmic origin of observed traits (Jinks 1961). In this test, two strains of varying characteristics are used. One strain carries an easily identifiable nuclear marker such as spore colour, while the other carries the phenotype of unknown origin (in this instance the *sec*− phenotype). The fused heterokaryon of these two strains is then resolved into its component homokaryons. If both homokaryons carry the unknown phenotype, it can be inferred that an involvement of cytoplasmic element(s) is likely. However, if the traits segregate in the individual monokaryons, nuclear control is likely. Using such tests, several well-known phenotypes associated with either aberrant mitochondrial genomes or plasmids or virus-like particles have been elucidated, including the 'mycelial, low sporulation' variant in *Penicillium* species and the

'purple, mycelial' variant in *A. nidulans* (Kale & Bennett 1992). In our study, upon subjecting each *sec*+ *x* *sec*− heterokaryon to a labourious analysis, every characteristic of the *sec*− phenotype (altered morphology, reduced sporulation and loss of secondary metabolite production) segregated together and remained associated with the *sec*− spore colour acting as the nuclear marker (data not shown). This suggested lack of involvement of extranuclear factor(s) in the formation and maintenance of the *sec*− variant.

The reproducible heterokaryon segregation data suggesting lack of involvement of cytoplasmic elements in the *sec*− phenotype led us to examine the regulation of the AF pathway itself, especially with respect to the regulatory gene *afl*R, whose product is a zinc binuclear cluster DNA-binding protein (Chang *et al*. 1993, Woloshuk *et al*. 1994). In our study, a comparison of the *afl*R coding region in the *sec*− and the *sec*+ strains revealed no change in the DNA sequence, ruling out point mutation or other structural changes in the *afl*R as the cause of the *sec*− phenotype.

Northern blots showed that the *sec*− strains produced *afl*R transcripts but in 5- to 10-fold lower levels compared to their *sec*+ parents (Fig. 2A). The RT–PCR results for the *afl*R gene further confirmed that *afl*R was being expressed in both the *sec*+ and the *sec*− strains (Fig. 2B). For the remaining three intron-containing genes (*omt*A, *ver-1*, and *nmt-1*), primers were designed such that the smaller cDNA product would be distinguished from the larger DNA-contamination product. As stated earlier in the Results section, the presence of only the cDNA band for the *nmt-1* gene in all four strains ruled out any possibility of genomic DNA contamination (Fig. 2B). In light of these results, the larger sized RT–PCR products for the *omt*A and *ver-1* genes in the sec− strains (Fig. 2B) suggest that these mRNAs are unprocessed (introns have not yet been spliced). Further, these results indicate that in the *sec*− strains, the levels of the *omt*A and *ver-1* transcripts were extremely low since they could not be detected in the 25 μg of total RNA in Northern hybridizations (Figs 2B and 2A respectively). It is possible that a threshold level of functional AFLR protein needs to be present in order to activate the expression of genes encoding the enzymes necessary to process aflatoxin pathway gene transcripts. A similar observation has been reported in the *afl*R knockout mutants of *A. parasiticus SU-1* (Cary *et al*. 2002) where it was suggested that a threshold level of AFLR protein must be reached in order to first activate itself and consequently to activate the AF pathway genes. Perhaps in the *sec*− variants the AFLR levels are not sufficient to activate its own expression as well as those of the other aflatoxin pathway genes. Further, since a positive regulatory region on the *afl*R promoter binding to both AFLR and an unknown protein has been identified (Ehrlich *et al*. 1999), another possibility is that an as yet unidentified factor(s) which normally interacts with AFLR to fully activate *afl*R expression is not produced or is produced in insufficient amounts in the *sec*− strains. This would explain the reduced levels of *afl*R transcript and, consequently, the extremely low levels of non-processed *ver-1* and *omt*A transcripts in the *sec*− strains. Considering the pleiotropic effects of the *sec*− strains on morphology, sporulation, and AF production, it is speculated that such a factor(s) may have a regulatory role in both fungal development and secondary metabolism. In this context, several recent reports on sterigmatocystin (ST)-producing *A. nidulans* have shown that elements other than the *afl*R gene regulate ST production. For example, a correlation between ST synthesis and sporulation was reported in *A. nidulans* (Guzman-de-Pena, Aguirre & Ruiz-Herrera 1998) where studies on developmental mutants indicated that this correlation occurred at a step prior to *brl*A, a gene shown to be necessary and sufficient to cause a switch from vegetative mycelial growth to conidiophore formation (Adams, Boylan & Timberlake 1988). Butchko *et al*. (1999) isolated two classes of *A. nidulans* mutants unable to make the secondary metabolite NOR (an intermediate of both AF and ST pathways). One group of mutants showed morphological defects while in the second group the morphology resembled the norsolorinic acid (NOR)+parent, indicating that there were factors distinct from both the *afl*R gene and the developmental genes that controlled ST production. Around the same time, these researchers discovered that both asexual sporulation and ST production in *A. nidulans* require inactivation of the proliferative growth through inhibition of the FadA (G protein) signalling pathway (Hicks *et al*. 1997). More recently, they have succeeded in identifying a gene called *pka*A encoding the catalytic subunit of the protein-phosphorylation enzyme protein kinase A, as another component of this signalling pathway (Shimizu & Keller 2001).

In the toxigenic *A. parasiticus*, a gene designated *flu*P and hypothesized to function in AF biosynthesis was cloned recently (Zhou, Rasooly & Linz 2000). Disruption of this gene resulted in an appearance of a 'fluffy' hyphal morphology, reduction or elimination of asexual spores and 2-fold reduction in AF accumulation, suggesting that it is associated with hyphal growth and development and that its activity indirectly affects AF accumulation (Zhou *et al*. 2000). These reports and recent reviews (Adams & Yu 1998; Calvo *et al*. 2002, Hicks, Shimizu & Keller 2002) reflect the highly complex nature of regulation of secondary metabolite production.

In summary, the *A. parasiticus sec*− forms were characterized using classical and molecular techniques. In parasexual crosses, the *sec*− forms failed to form heterokaryons and diploids with other *sec*− strains. The heterokaryon test indicated any involvement of cytoplasmic elements such as mitochondria, plasmids or mycoviruses in this phenomenon was unlikely. On a molecular level, the *sec*− forms expressed the *afl*R

regulatory gene but at a level 5- to 10-fold lower than their *sec*+ parents. Representative AF structural genes, *omt*A and *ver-1* were also expressed by the *sec*− variants, but in such low amounts that they were detected only by RT–PCR. These results add to the growing body of data that indicate that in the aflatoxigenic fungi, *afl*R is necessary but not sufficient for the expression of the AF pathway. Further studies using the isogenic *sec*− and *sec*+ strains should uncover the transcriptional and/or translational events responsible for the characteristics of this unique group of non-toxigenic variants.

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